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ORIGINAL ARTICLE

Gestational weight gain by reduced brain melanocortin activity affects offspring energy balance in rats

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Introduction: Excessive gestational body weight gain of mothers may predispose offspring towards obesity and metabolic derangements. It is difficult to discern the effects of maternal obesogenic factors—such as diet and/or thrifty genetic predisposition—from gestational weight gain *per se*.
Methods: For this reason, genetically normal Wistar rats that were fed regular chow were rendered hypothalamically obese by chronic third-cerebral ventricular (i3vt) infusion during pregnancy and lactation with the melanocortin-3,4 receptor blocker SHU9119. This procedure caused significant increases in body weight gain during pregnancy and lactation compared with controls, and the effects thereof on offspring energy balance and fuel homeostasis were investigated.
Results: At birth, litter weight and size, but not individual pup weight, of SHU9119-treated mothers were significantly smaller than controls. In litters culled to eight, pup weight gain during lactation was only transiently increased by treatment. After weaning, however, male offspring of SHU9119-treated mothers became increasingly heavier over time relative to controls until killing at 9 months. This effect was only transient in females. Increased body weights of males were not associated with disturbances in glucose homeostasis, but with increased energy expenditure instead. Multiple regression analysis revealed that gestational body weight gain, irrespective of the group, contributed positively to increased visceral fat deposition and carbohydrate oxidation in the male offspring. In contrast, the pre-pregnancy body weight of mothers contributed positively to male offspring daily energy expenditure, subcutaneous fat and eviscerated carcass as well as structural organ weights. In female offspring, gestational body weight gain, but not pre-gestational body weight, contributed both to aspects of weight gain as well as to the shift of fat oxidation toward carbohydrate oxidation.
Conclusion: Gestational weight gain induced by low brain melanocortin receptor activity can lead to increased body weight gain in the offspring (particularly in males) independent of obesogenic dietary and/or thrifty genetic predisposition.
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Keywords: pregnancy; lactation; SHU9119; maternal obesity; generational effects; metabolic imprinting

Introduction

According to the report of a joint WHO/FAO expert consultation in 2003, over 1 billion people are estimated to be overweight,[1] and conditions associated with obesity—for example, type II diabetes mellitus, cardiovascular diseases and certain types of cancer—have devastating health consequences. If no actions are taken, the economic consequences to societies will be staggering in the near future.[2,3] The contributing factors are probably a sedentary lifestyle and changes in dietary habits,[4] particularly in genetically predisposed obese individuals.[5] Obesity-prone individuals may differ from obesity-resistant ones because of the reduced efficacy with which the adipocyte hormone leptin interacts with neuronal networks involved in the control of ingestive behaviour, neuroendocrine outflow and metabolism.[6–8] A key network involved in the signalling of leptin is the brain melanocortin (MC) system.[9] Under *ad libitum* feeding, non-obese conditions, leptin stimulates arcuate nucleus (ARC) neurons that synthesize and secrete α-melanocyte stimulating hormone, and reduces the activity of ARC neurons that synthesize and secrete agouti-related peptide (AgRP).[10,11] The latter neurons contain the

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orexigenic neuropeptide Y (NPY) whose synthesis and secretion are also depressed by leptin .[12] α-MSH acts agonistically and AgRP inverse agonistically on brain MC receptors, which together underlie reduced ingestive behaviour and increased metabolic rate.[13] The idea that MC receptors are downstream from leptin signalling comes from studies in which the third-intracerebroventricular (i3vt) effects of leptin could be blocked by the cyclic α-MSH analogue SHU9119,[14] which is a strong antagonist of MC3/4 receptors.[15] Continued i3vt treatment with SHU9119 has been shown to cause obesity in rats.[9,16] Leptin resistance caused by polymorphisms in MC-associated genes constitutes the most prevalent monogenic causes of obesity known today.[17,18]

Obesity is not caused only by interaction between genes and the traditional adult risk factors. It is becoming increasingly clear that the interplay between genes and the embryonic, fetal and early postnatal environment is also of tremendous importance. Although initial observations on humans focused on the relationship between low birth weight and risk of adult obesity and metabolic syndrome,[19] mothers who are overweight during pregnancy have children with high risk of increased birth weight and/or adiposity.[20] In turn, this can increase the risk for childhood and adult obesity.[21] It is difficult, however, to discern the direct effects of gestational obesity from dietary or genetic factors that drive the obese state in the offspring. To study the effects of gestational weight gain on offspring body weight and on the factors related to fuel homeostasis independent of genetic predisposition or diet, we first aimed at introducing increased weight gain in rats during gestation and lactation by i3vt SHU9119 infusion in a dose earlier found to be effective in male rats.[9,16] It was already known from the work of Polidori and Geary[22] that female rats are equally sensitive to the orexigenic actions of central SHU9119 treatment, as compared with male rats, and that oestrogen does not affect the sensitivity to SHU9119, but no studies have been performed to assess the effects of SHU9119 treatment on body weight gain during pregnancy and lactation. If reduced brain MC activity during pregnancy and lactation causes increased weight gain in these animals as well as in their offspring, the implication could be that polymorphisms in genes that inhibit the activity of the brain MC system may –besides being passed on genetically—also provide a perinatal environment leading to increased weight gain and disturbances in fuel homeostasis in the offspring through a non-genetic route.

Methods

Housing

All the rats used in these experiments were from the Wistar strain and obtained from Harlan Netherlands (Horst). They were initially group-housed in our animal rooms at room temperature (20 ± 2 °C) on a 12:12 light–dark cycle (lights off at 1400 h), with food (RMHB-laboratory chow, Charles River) and water available *ad libitum*. All methods were approved by the Local Ethics Committee of the University of Groningen.

Pregnancy and lactation

After a habituation period of several days in our animal quarters, 20 female rats (270–290 g, between 4–5 months of age) were anesthetized under isoflurane/N_2O inhalation and implanted stereotaxically with a 22 G stainless steel guide cannula (Plastics One, Roanoke, VA, USA) aimed i3vt.[23] After surgery, each rat was given 1.0 mg kg^{-1} flunixin–meglumin s.c. (Schering-Plough, Maarssen, The Netherlands) for analgesia, and housed individually in a plastic cage (lXbXh: 25 × 25 × 35 cm) with clean wood shavings and shredded cardboard. After recovery from surgery, correct cannula placement was confirmed by induction of a drinking response following i3vt injection of 10 ng of angiotensin-II.[23] Twenty male rats in separate cages were housed in the same room to stimulate oestrus cycling in the female rats, which was assessed by taking daily vaginal smears just before the start of the dark period. On confirmation of cyclicity for at least four cycles, each female was housed together with a male for one day during the end of the pro-oestrus or the beginning of oestrus. Copulation was confirmed by the presence of vaginal plugs and/or the presence of sperm cells in the vaginal smears (day 1 of pregnancy). Maternal body weights were assessed until day 25 post-delivery. Because pups started to ingest food and drink water after day 16 of lactation, no food and water intake recordings are shown beyond that day.

At day 4 of presumed pregnancies, animals were divided into two groups that had approximately the same mean body weight (290.2 ± 5.3 vs 296.5 ± 5.5 g, $P = 0.403$). Under isoflurane inhalation anaesthesia (N_2O was excluded because of its reported risk in inducing abortion[24,25]), each rat had an osmotic minipump (Alzet 2004, pumping rate 0.25 μl h^{-1} over 28 days; Alza, Palo Alto, CA, USA) implanted subcutaneously. These minipumps were connected with polyethylene tubing (PE50) to an injector placed permanently into the previously positioned i3vt cannula. In one group ($n = 8$), pump and connector tubings were filled to deliver sterile saline. In the other group ($n = 12$), pumps and connector tubings were filled to deliver SHU9119 ((Ac-Nle4, Asp5, D-2-Nal7, Lys10)-cyclo-α-MSH$^{4-10}$ amide, M-4603; Sigma-Aldrich Chemie, Sternheim, Germany, 0.5 nmol in sterile saline per day). This dose of SHU9119 causes hyperphagia and obesity in adult male Wistar rats.[9] Eight out of 12 SHU9119-treated rats (67%) and five out of eight saline-treated rats (63%) became pregnant. Two SHU9119-treated mothers were excluded as they developed inflammation around the minipump during a late stage of pregnancy and started to lose weight gradually during lactation. Non-pregnant SHU9119-treated ($n = 4$) and saline-treated rats ($n = 3$) served as non-pregnant controls. One additional non-operated and non-pregnant control group ($n = 3$) was added to the study to assess the effects of saline treatment

per se. As the body weight and ingestion parameters were identical among saline-treated and non-treated controls, we decided to group them as 'non-pregnant' controls ($n=6$).

On the day of birth, litters of healthy mothers were weighed, counted and equalized to eight. In the saline-treated group, litter counts ranged between 7 and 14. The litter of seven pups was substituted by a surplus pup from a large litter. In the SHU9119-treated group, litter counts ranged from 7 to 10. One whole litter was divided among others, as the number of surplus pups was insufficient. The 'cross-fostered' pups in the saline and SHU9119 groups were toe-clipped and not used for further analysis. At day 17 of lactation, one litter from a saline-treated and from an SHU9119-treated mother was terminated to assess the biochemical and anatomical characteristics, leaving four litters per group for offspring analysis after weaning. Except for individual pup weight registration at day 16, the remaining litters were left undisturbed until weaning. At day 25, mothers were killed by carbon dioxide exposure after which body composition analysis was performed. Intestines, liver, spleen, adrenals, kidneys, thymus, retroperitoneal fat, subcutaneous fat (including skin) and muscle (stripped carcass without subcutaneous fat and skin) were all removed and weighed separately.

Offspring

Metabolic and endocrine parameters. After weaning, offspring rats were housed in numbers of two 2–3 in type IV cages with males and females separated, mixed among litters, and weighed at 1, 2, 3, 6 and 9 months of age. Basal fuel and hormone levels were assessed at 2 months of age by tail bleeding (0900–1000 h). Samples (500 μl) were collected within 1 min into chilled tubes (0 °C) containing 0.05 g ml^{-1} EDTA. All blood samples mentioned here and elsewhere were centrifuged for 15 min. at 1500 *g*, 4 °C. Plasma samples were stored at −20 °C until analysis for insulin, leptin, adiponectin and glucose content. Glucose levels were measured by the ferricyanide method of Hoffman[26] (Technicon Auto Analyzer TMII). Plasma levels of insulin (Linco kit no. RI-13K), leptin (Linco kit no. RL-83K) and adiponectin (Linco kit no. MADP-60HK) were determined by commercial assays. After blood sampling at 2 months, offspring rats were put individually in cages for 10 days to measure food and water intake. Between days 2 and 9 of this individual housing period, animals were placed individually during a 26 h period in a respirometric chamber (l × b × h: 25 × 35 × 25 cm), with food and water available *ad libitum*. After habituation for 2 h, oxygen consumption (V_{O_2}, l h^{-1}) and carbon dioxide production (V_{CO_2}, l h^{-1}) were recorded, and carbohydrate oxidation (CHO-ox, mg min^{-1}), fat oxidation (FAT-ox, mg min^{-1}) [27]and metabolic rate (MR, kJ h^{-1})[28] were calculated. Resting metabolic rate (RMR, kJ h^{-1}) was defined as the lowest (running) mean metabolic rate (MR) recorded over half an hour anywhere during the 24-h measurement. MR over 24 h was used to calculate the daily energy expenditure (DEE, kJ d^{-1}).

Glucose homeostasis. To investigate the effects of maternal SHU9119 treatment versus saline treatment during pregnancy and lactation on offspring glucose homeostasis, an oral glucose tolerance test (OGTT) at 3 months as well as an intravenous glucose tolerance test (IVGTT) at 7 months was performed.

OGTT: Animals were trained over 1 week to swallow a gavage tube (outer diameter: 1.5 mm) that could be advanced in the esophagus for gastric injections. During the night before the experiment, animals were individually housed and received 6 g of food (semi-fasted), with water available *ad libitum*. On the next morning, a basal blood sample (150 μl) was taken by tail bleeding within 1 min, and the animals received 1 g of glucose in 5 ml water by gavage and were put back into their cage. Further blood samples were taken by tail bleeding at 15, 30 and 45 min. In all samples, the levels of glucose and insulin were assessed in plasma by the abovementioned methodology.

IVGTT: At 6.5 months of age, animals were housed individually (l × b × h: 25 × 25 × 35 cm) and surgically equipped under isoflurane/N_2O inhalation anaesthesia with silicon cannulas in the right and left jugular veins according to methods described earlier.[29] The cannulas were filled with 55% polyvinylpyrrolidone in heparinized water (400 U ml^{-1}) to maintain patency between surgery and experimental blood sampling. Animals were injected with 1.0 mg kg^{-1} flunixin–meglumin (s.c.) (Schering-Plough, Maarssen, The Netherlands) for analgesia after surgery. After recovery from surgery, animals were habituated three times to blood sampling during the middle of the light phase. Two hours before the actual IVGTT, animals were food-deprived and connected to tubing for remote and stress-free blood sampling and infusion. Two basal blood samples (150 μl) were taken after which ($t=0$) a glucose infusion was started (10 mg min^{-1} in 2 ml saline over 20 min). Blood samples for determination of plasma glucose and insulin levels were taken at $t=1$, 3, 5, 7, 10, 15, 20, 23, 26, 30, 40 and 50 min. Two weeks later, females from one litter were taken out of the experiment because of inflammation around the jugular vein cannulas.

Carcass analysis. At 9 months, all animals from the remaining litters were decapitated after short CO_2 exposure for body composition analysis. Blood was collected for end point fuel and hormone analysis as described above. The following organs were removed and weighed immediately: intestines, liver, spleen, adrenals, kidneys, thymus, retroperitoneal fat, epididymal fat, subcutaneous fat (including skin) and muscle (stripped carcass without skin).

Statistical analysis

Maternal body weight, food and water intake were analyzed using repeated measures ANOVA with treatment as between

subject factor. The effects of SHU9119 versus saline treatment on carcass parameters of mothers and litter characteristics were analyzed using *t*-tests. The effects of SHU9119 treatment during pregnancy relative to control treatment on offspring growth curves (after natural log transformation) were analyzed with General Linear Models using a repeated measures design in which litters were nested in treatments and litter was designated as a random factor. Individual blood glucose and plasma insulin responses during the OGTT and IVGTT experiments were calculated by area under the curve (AUC). Basal fuel and hormone levels, metabolic parameters, ingestive parameters, glucose homeostasis (based on AUCs) and body composition were analyzed with General Linear Models with nested design as mentioned above, and the litter designated as a random factor. All results from male and female offspring were analyzed separately. Multiple regression analysis was performed to investigate how variation in peri-gestational weight parameters of mothers and their treatment contributed to variation in various parameters related to body weight and fuel homeostasis in the offspring. In this event, scores from animals per gender were averaged within each litter. In all tests, $P<0.05$ was considered significant. Data are presented as averages ± s.e.m. All statistical analyses were performed using SPSS 15.0 for Windows.

Results

Effects of SHU9119 and saline treatment on pregnancy and lactation

ANOVA detected significant differences in food intake over time during pregnancy ($F(17,153)=8.98$; $P<0.0001$) and lactation ($F(16,128)=38.70$; $P<0.0001$) (Figure 1a) compared with non-pregnant controls. Food intake responses were stronger over time in SHU9119-treated (though not significantly different from SHU9119-treated non-pregnant rats) than in saline-treated females during pregnancy (time × treatment: $F(17,153)=2.74$; $P=0.001$). During lactation, however, differences in food intake between SHU9119-treated and control-treated rats were lost (time × treatment: $F(16,128)=1.36$; $P=0.17$). In addition, water intake increased significantly over time during pregnancy ($F(17,153)=8.49$; $P<0.0001$) and lactation ($F(16,128)=23.24$; $P<0.0001$) (Figure 1b), and increases during pregnancy were again more pronounced in SHU9119-treated than in saline-treated females (time × treatment: $F(17,153)=2.53$; $P=0.001$). No interaction was found during lactation (time × treatment: $F(16,128)=1.05$; $P=0.40$). Body weight increased significantly over time during pregnancy ($F(20,180)=95.46$; $P<0.001$) and lactation ($F(25,200)=3.99$; $P<0.001$) (Figure 1c). During pregnancy, body weight increase was more pronounced in SHU9119-treated than in saline-treated females (time × treatment: $F(20,180)=5.36$; $P<0.0001$). During lactation, no interaction effect was found $F((25,200)=1.53$; $P=0.057$). Relative to the pre-gestational body weight, body weight gain of SHU9119-treated females was 177 ± 27 g (+59% increase), which was significantly larger ($F(1,9)=5.689$, $P=0.041$) than that of saline-treated females (increase of 109 ± 10 g, +38%). At parturition, maternal drop in body weight was different between SHU9119-treated (58 ± 6 g, 12%) and saline-treated (88.6 ± 10 g, 22%) females ($F(1,9)=8.77$; $P=0.016$). During lactation SHU9119-treated females were still heavier than saline-treated females ($F(1,8)=8.96$; $P=0.017$). Non-pregnant SHU9119-treated females continued to eat more than the

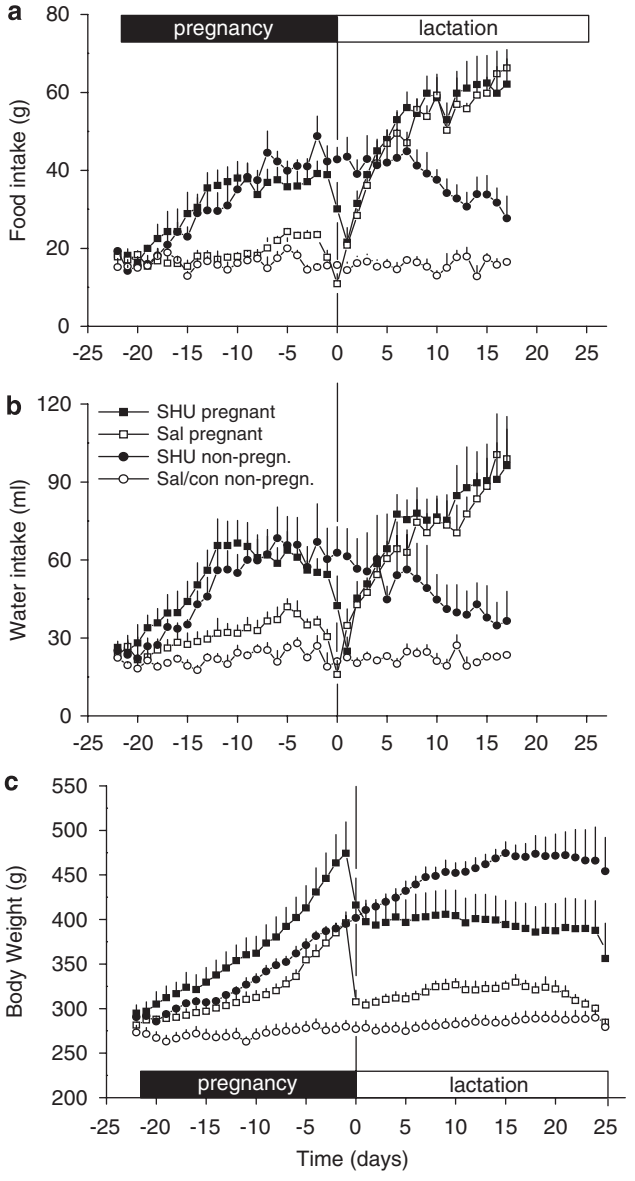

Figure 1 Food intake (**a**), water intake (**b**), and body weight (**c**) of female rats during pregnancy and lactation and their respective controls are depicted. Treatments include i3vt infusion in pregnant/lactating rats of the melanocortin blocker SHU9119 (SHU pregnant, $n=6$, but $n=5$ from day 1 of lactation) and saline (Sal pregnant, $n=5$), and i3vt infusion in non-pregnant/non-lactating rats of SHU9119 (SHU non-pregnant, $n=4$) and saline or no infusion (Sal/con non-pregnant, $n=6$). Data are expressed as mean ± s.e.m.

normal 20 g day^{-1} until the end of the infusion period (at approximately day 10 of lactation in the lactating animals) compared with non-pregnant controls, resulting in continued increased body weight.

Litter and mother characteristics

Table 1 shows characteristics of litters from SHU9119-treated and saline-treated mothers on the day of birth. Litters from SHU9119-treated mothers were significantly lighter than those from saline-treated mothers ($P=0.006$), which was partly explained by the smaller litter size ($P=0.043$). There was no difference in individual pup weight at birth ($P=0.727$). Dead/alive ratios ($P=0.204$) and male/female ratios ($P=0.551$) were similar in SHU9119- and saline litters. At 16 days of age, pups in litters of SHU9119-treated mothers were significantly heavier ($P=0.022$), but this difference disappeared at weaning at 25 days of age ($P=0.790$). At day 25 of lactation, mothers were killed by decapitation for examination of body composition (Table 2), and the body weight of SHU9119-treated mothers was higher than that of saline-treated mothers ($P=0.005$). Adiposity was increased in SHU9119-treated mothers, as indicated by their significantly heavier retroperitoneal ($P=0.001$) and subcutaneous fat ($P=0.002$) pads. In addition, liver ($P=0.005$), gastrointestinal tract ($P=0.013$), kidneys ($P=0.016$) and thymus ($P=0.002$) were heavier in SHU9119-treated mothers. No differences between treatment groups were observed in the spleen, adrenals and muscle weight.

Effect of maternal SHU9119 and saline treatment on offspring body weight

Offspring body weights from saline-treated and SHU9119-treated mothers from weaning until 9 months of age are shown in Table 3. General Linear Models with time and treatment (in which litters were nested) as factors revealed a significant interaction effect in male offspring ($B=13.50$, s.e. $=5.73$, $\chi^2=5.55$, $P=0.018$), and only a treatment effect in females ($B=18.89$, s.e. $=9.11$, $\chi^2=4.30$, $P=0.038$). Body weights of male offspring from SHU9119-treated mothers were higher than those from saline-treated mothers from the third month until decapitation at 9 months of age. Body weight of female offspring from SHU9119-treated mothers were only transiently increased relative to female offspring from saline-treated mothers, that is, from the second until the sixth month of age.

Table 1 Litter characteristics of SHU9119- and saline-treated mothers

Litters from	*Saline-treated mothers*	*SHU9119-treated mothers*
Litter size	11.0 (1.4)	7.3 (1.1)*
Litter weight (g)	76.3 (3.9)	49.18 (6.92)**
Dead/alive ratio	0	0.089 (0.064)
Male/female ratio	1.27 (0.39)	0.91 (0.48)
Pups still alive at day 1	11.0 (1.4)	6.7 (1.1)*
Pup birth weight (g)	6.46 (0.26)	6.55 (0.11)
Pup weight 16 days (g)	37.9 (1.7)	47.2 (3.2)*
Pup weight 25 days (g)	95.5 (6.4)	98.3 (5.8)

Pups were counted and weighed individually upon finding the litter at day 0 of lactation. Characteristics of litters at birth of healthy mothers only were analyzed (saline: $n=5$, SHU9119: $n=6$). After counting the surviving pups at day 1, litters were equalized to eight. One litter from a SHU9119-treated mother was divided over the other litters from SHU9119-treated mothers, because surplus pups were insufficient to equalize all litters to eight pups. Therefore, results at day 16 are based on five saline and five SHU9119 litters. One litter of each group was terminated on day 17 of lactation to assess biochemical and anatomical characteristics, leaving four litters from saline-treated mothers and four litters from SHU9119-treated mothers at day 25. Asterisks denote significant difference (*$P<0.05$; **$P<0.01$).

Table 2 Whole body and organ weights presented as averages (± s.e.m.) after i3vt SHU9119- or saline treatment during pregnancy and lactation

	Saline-treated mothers (n = 4)	*SHU9119-treated mothers (n = 4)*
Body weight (g)	289.4 (10.7)	406.3 (29.3)**
Liver (g)	12.1 (1.1)	19.2 (2.1)*
Gastrointestinal tract (full) (g)	28.3 (4.2)	50.8 (6.0)*
Spleen (g)	0.69 (0.04)	0.82 (0.10)
Kidneys (g)	2.33 (0.07)	3.06 (0.24)*
Thymus (g)	0.196 (0.019)	0.481 (0.060)**
Adrenals (g)	0.084 (0.010)	0.114 (0.012)
Subcutaneous fat (g)	44.8 (2.4)	83.9 (8.3)**
Retroperitoneal fat (g)	5.9 (1.1)	30.3 (4.6)**
Carcass (g)	164.9 (5.2)	178.9 (5.0)

Body composition was assessed in healthy mothers that successfully reared their pups to weaning. Asterisks denote significant difference (*$P<0.05$; **$P<0.01$).

Effect of maternal SHU9119 and saline treatment on offspring metabolic and ingestive parameters

Using General Linear Models with treatment (in which litters were nested) as factor revealed that neither the assessed plasma hormone and fuel levels nor the ingestive parameters were different between offspring groups (Table 4). However, RMR ($F(1,21)=54.238$, $P=0.041$) and DEE ($(F1,21)=5.443$, $P=0.046$) in male offspring from SHU9119-treated mothers were 12.5 and 7.6%, respectively, higher than in male offspring from saline-treated mothers. CHO oxidation tended to be increased in male offspring from SHU9119-treated mothers relative to controls ($F(1,21)=4.306$, $P=0.066$). Only trends were observed in females (Table 5). The differences in weight observed above were not associated with deteriorations in glucose homeostasis, as indicated by the similar outcomes of the OGTT and IVGTT.

Multiple regression analysis

Although maternal treatment effects on offspring body composition and glucose homeostasis failed to reach statistical significance, there were in almost all assessed parameters strong effects of litter in the nested design

Table 3 Treatment effects of i3vt SHU9119 versus saline during pregnancy and lactation on offspring body weight presented as averages (±s.e.m.)

Offspring (based on individual scores) from	*Saline-treated mothers*	*SHU9119-treated mothers*	*PGBW*	*GBWG*	*LW and/or GP*
Males					
1 month (g)	104.7 (2.4)	100.0 (3.0)	—	—	—
2 months (g)	301.4 (4.1)	319.1 (6.5)*	—	—	—
3 months (g)	417.4 (5.2)	463.0 (10.5)***	↑, $P=0.049$	—	—
6 months (g)	521.2 (6.2)	555.9 (12.3)**	—	—	—
9 months (g)	560.8 (9.8)	600.7 (12.5)*	↑, $P=0.018$	—	—
Females					—
1 month (g)	94.7 (2.8)	95.9 (2.7)	—	—	—
2 months (g)	208.0 (4.3)	223.3 (3.2)**	—	—	GP, $P=0.01$
3 months (g)	260.3 (6.1)	281.7 (4.0)**	—	—	GP, $P=0.04$
6 months (g)	293.1 (7.8)	319.3 (6.7)*	—	—	—
9 months (g)	325.1 (6.8)	344.4 (7.4)	—	↑, $P=0.001$	GP, $P=0.001$

Asterisks denote significant difference (*$P<0.05$; **$P<0.01$, ***$P<0.001$), and multiple regression analysis with pre-gestational body weight (PGBW), gestational body weight gain (GBWG), and litter weight (LW) and/or group (GP) as factors. ↑,↓ indicates direction of interaction.

Table 4 Treatment effects of i3vt SHU9119 versus saline during pregnancy and lactation on male offspring characteristics are presented as averages (±s.e.m.)

Male offspring	*Saline-treated mothers*	*SHU9119-treated mothers*	*PGBW*	*GBWG*	*LW and/or GP*
Fuel and hormones 2 months					
Glucose (mM)	6.33 (0.09)	7.08 (0.20)	—	↑, $P=0.005$	—
Insulin (ng ml^{-1})	2.56 (0.16)	3.04 (0.43)	—	↑, $P=0.011$	—
Leptin (ng ml^{-1})	4.34 (0.26)	5.16 (0.69)	—	—	—
Adiponectin (ng ml^{-1})	3378 (138)	4064 (201)	—	—	—
Fuel and hormones 9 months					
Glucose (mM)	6.28 (0,12)	6.36 (0.18)	—	↑, $P=0.006$	GP, $P=0.004$ LW, $P=0.001$
Insulin (ng ml^{-1})	3.47 (0,34)	2.93 (0.44)	—	—	—
Leptin (ng ml^{-1})	5.86 (0.70)	6.08 (0.36)	—	—	—
Adiponectin (ng ml^{-1})	2052 (139)	2421(146)	—	—	—
Food and water intake					
Food intake (g day^{-1})	22.58 (0.81)	23.01 (0.57)	—	—	—
Water intake (g day^{-1})	29.45 (1.98)	33.8 (1.4)	—	—	—
Substrate metabolism					
CHOox (mg min^{-1})	1051 (37)	1183 (25)	—	↑, $P=0.018$	—
FATox (mg min^{-1})	1338 (150)	1171 (79)	—	—	—
RMR (kJ h^{-1})	8.19 (0.24)	9.20 (0.24)*	—	—	—
DEE (kJ day^{-1})	264.8 (3.4)	284.8 (5.6)*	↑, $P=0.015$	—	—
OGTT					
AUC (0–45) glucose	290.3 (4.1)	314.6 (10.3)	↑, $P<0.0001$	—	—
AUC (0–45) insulin	231.6 (8.2)	251.9 (23.5)	↑, $P=0.045$	—	—
IVGTT					
AUC (0–50) glucose	320.2 (7.3)	320.5 (7.5)	—	—	—
AUC (0–50) insulin	168.6 (13.0)	151.4 (12.4)	—	—	—
Body composition					
Liver (g)	20.4 (0.4)	21.1 (0.6)	↑, $P=0.034$	—	—
Intestines (g)	33.3 (0.7)	33.0 (0.6)	—	—	—
Spleen (g)	1.02 (0.05)	1.13 (0.07)	—	—	—
Kidneys (g)	3.33 (0.13)	3.88 (0.16)	—	—	—
Thymus (g)	0.37 (0.02)	0.48 (0.05)	—	↑, $P=0.039$	—
Adrenals (g)	0.084 (0.003)	0.078 (0.004)	—	—	—
Subcutaneous fat (g)	102.7 (3.4)	112.4 (4.6)	↑, $P=0.028$	—	—
Retroperitoneal fat (g)	7.33 (0.60)	10.0 (1.4)	—	↑, $P=0.013$	GP, $P=0.077$
Epididymal fat (g)	16.9 (0.7)	19.3 (1.0)	—	↑, $P=0.039$	—
Carcass (g)	328.4 (6.1)	350.7 (6.7)	↑, $P=0.04$	—	—

Results are based on nested design analysis (*$P<0.05$), and multiple regression analysis with pre-gestational body weight (PGBW), gestational body weight gain (GBWG), and litter weight (LW) and/or group (GP) as factors. ↑,↓ indicates direction of interaction.

Table 5 Treatment effects of i3vt SHU9119 versus saline during pregnancy and lactation on female offspring characteristics are presented as averages (± s.e.m.)

Female offspring	*Saline-treated mothers*	*SHU9119-treated mothers*	*PGBW*	*GBWG*	*LW and/or GP*
Fuel and hormones 2 months					
Glucose (mM)	5.85 (0.11)	3.09 (0.06)	—	—	—
Insulin (ng ml^{-1})	1.64 (0.10)	2.20 (0.14)	—	—	—
Leptin (ng ml^{-1})	2.52 (0.12)	2.81 (0.24)	↑, $P=0.026$	—	—
Adiponectin (ng ml^{-1})	4013 (324)	3964 (118)	—	—	—
Fuel and hormones 9 months					
Glucose (mM)	5.66 (0,21)	5.85 (0.17)	↑, $P=0.002$	—	—
Insulin (ng ml^{-1})	2.30 (0.40)	3.14 (0.48)	—	—	—
Leptin (ng ml^{-1})	3.53 (0.63)	3.04 (0.39)	—	—	—
Adiponectin (ng ml^{-1})	2619 (373)	3151(233)	—	—	—
Food and water intake					
Food intake (g day^{-1})	16.0 (0.9)	16.7 (0.8)	—	—	—
Water intake (g day^{-1})	23.8 (1.6)	25.9 (1.6)	—	—	—
Substrate metabolism					
CHOox (mg min^{-1})	795 (34)	835 (25)	—	↑, $P=0.007$	—
FATox (mg min^{-1})	945 (112)	831 (105)	—	↓, $P=0.004$	—
RMR (kJ h^{-1})	6.10 (0.14)	6.06 (0.15)	—	—	—
DEE (kJ day^{-1})	198.6 (3.4)	200.7 (3.9)	—	↑, $P=0.052$	—
OGTT					
AUC (0–45) glucose	302.2 (12.5)	305.7 (6.7)	—	—	—
AUC (0–45) insulin	238.7 (22.3)	293.2 (22.9)	—	—	—
IVGTT					
AUC (0–50) glucose	338.3 (7.8)	338.0 (14.0)	—	—	—
AUC (0–50) insulin	181.5 (19.0)	134.6 (14.0)	—	—	—
Body composition					
Liver (g)	10.4 (0.5)	12.0 (0.5)	—	—	—
Intestines (g)	22.0 (1.2)	26.0 (1.5)	—	—	—
Spleen (g)	0.74 (0.05)	0.90 (0.05)	—	—	—
Kidneys (g)	2.41 (0.10)	2.77 (0.15)	—	—	—
Thymus (g)	0.36 (0.08)	0.45 (0.05)	—	—	—
Adrenals (g)	0.089 (0.003)	0.112 (0.008)	—	—	—
Subcutaneous fat (g)	51.7 (2.6)	54.7 (2.0)	—	↑, $P=0.009$	—
Retroperitoneal fat (g)	13.0 (1.7)	15.8 (1.4)	—		—
Carcass (g)	191.5 (3.6)	196.8 (5.3)	—	↑, $P=0.018$	—

Results are based on nested design analysis, and multiple regression analysis with pre-gestational body weight (PGBW), gestational body weight gain (GBWG), and litter weight (LW) and/or group (GP) as factors. ↑,↓ indicates direction of interaction.

analysis. This means that individual mother–litter interactions were relatively large compared with the effects of SHU9119 versus saline treatment on offspring characteristics. Therefore, we performed multiple regression analysis in which (1) body weight of dams at day 0 of pregnancy (pre-gestational body weight), (2) body weight gain during pregnancy (gestational body weight gain) and (3) treatment group were included as independent factors. Using backward multiple regression analysis, these factors were analyzed on their relative significance in explaining variation in various offspring characteristics averaged per litter (dependent factors). Only when a significant interaction was found of gestational body weight gain and a relevant parameter in the offspring did we include litter weight as a factor. This procedure was chosen as litter weight is by definition a major component of body weight gain during pregnancy, but it may not necessarily be related to pre-gestational body weight. Indeed, pre-gestational body weight was significantly correlated neither with gestational body weight gain nor with litter weight.

In male offspring (Table 4), gestational body weight gain of mothers contributed in a positive direction to plasma glucose ($F(1,7)=18.93$) and plasma insulin ($F(1,7)=12.80$) at 2 months of age, plasma glucose at 9 months of age ($F(2,7)=16.8$, treatment group contributed as well), CHO oxidation ($F(1,7)=10.33$), and to the end point weights of the thymus ($F(1,7)=6.953$), retroperitoneal fat ($F(2,7)=8.03$; near-significant treatment effect as well), and epididymal fat ($F(1,7)=6.95$). Only plasma glucose at 9 months was affected more strongly by litter weight ($F(2,7)=39.21$) than by gestational weight gain *per se*. The pre-gestational body weight of mothers contributed in a positive direction to body weight at 3 months ($F(1,7)=6.05$) and 9 months ($F(1,7)=10.45$) of age (Table 3), to DEE

($F(1,7) = 11.23$), to the AUC of glucose ($F(1,7) = 60.15$) and insulin ($F(1,7) = 6.40$) in the OGTT, and to the end point weights of the liver ($F(1,7) = 7.54$), subcutaneous fat mass ($F(1,7) = 8.29$) and deskinned-eviscerated carcasses ($F(1,7) = 6.81$) (Table 4).

In females, a different picture emerged. As opposed to male offspring, female offspring body weight at 2 ($F(1,7) = 13.49$) and 3 ($F(1,7) = 6.86$) months of age was affected by the treatment group, but at 9 months the gestational body weight gain also became a significant factor ($F(2,6) = 54.63$) in a positive direction (Table 3). With respect to the metabolic parameters (Table 5), the gestational body weight gain of mothers contributed positively to CHO oxidation ($F(1,7) = 16.059$) and inversely to Fat oxidation ($F(1,7) = 21.75$). There was a trend for gestational body weight gain to affect DEE ($F(1,7) = 5.65$) in a positive direction. Gestational body weight gain (but not pre-gestational body weight as was found in males) contributed to the weight of subcutaneous fat mass ($F(1,6) = 17.58$) and the deskinned-eviscerated carcass ($F(1,6) = 12.05$) in a positive direction. In none of the cases did litter weight contribute significantly. The pre-gestational body weight of mothers significantly affected plasma leptin levels only at 2 months of age ($F(1,7) = 8.58$) and plasma glucose at 9 months of age ($F(1,6) = 38.41$).

Discussion

The general aim of these experiments was to investigate the effects of gestational weight gain in rats on several parameters related to energy balance and fuel homeostasis in the offspring. For this purpose, we first assessed whether pregnant rats were able to increase ingestive behaviour and body weight, and still were able to produce viable offspring on inhibition of brain melanocortin (MC) signalling by chronic i3vt infusion of the MC3/MC4 receptor antagonist SHU9119 during pregnancy and (part of) lactation. This situation is very relevant for humans as reduced activation of brain MC receptors, leading to gestational weight gain, can be the result of not only genetic[30] but also probably hormonal[31] and dietary[32] causes. As expected, we observed increases in food and water intake in pregnant and particularly lactating rats, and these are regarded as appropriate responses necessary to meet the energetic requirements of developing offspring. Both food and water intake were markedly enhanced during pregnancy by i3vt SHU9119 treatment and therefore caused a strong increase in body weight during pregnancy in these animals. Although Grattan *et al*.[33] previously reported leptin resistance during the second phase of pregnancy, this effect is probably not due to downregulation of MC4 receptors or their downstream signalling components.

A different picture emerged during lactation, where i3vt SHU9119 treatment was not effective in increasing food and water intake above the augmented levels found in vehicle-treated controls. The most direct explanation for this inability is that the levels of food and water intake had reached a certain physiological barrier. Artificially increasing the litter size[34] or concurrent pregnancy[35] does not further augment lactation-induced hyperphagia beyond a certain level either.[35] Johnson *et al*.[36] argued that this physiological barrier may be related to maximal heat dissipation of mothers to avoid hyperthermia, as increasing heat dissipation by cold exposure or removal of fur from the back of the lactating mother was able to further increase food intake beyond the pre-existing barrier. It seems likely that, under these 'maximized' intake conditions, MC4 receptor signalling is already completely abolished , leaving nothing left to block with SHU9119. In line with this observation is the report by Crowley *et al*.[37,38] that mRNA expression of AgRP in the ARC is strongly upregulated, which is probably mediated by suckling. AgRP may lead to maximal suppression of brain MC receptor signalling, rendering additional pharmacological inhibition of brain MC receptors ineffective.

Another relevant comparison can be made between pregnant and non-pregnant groups, both i3vt treated with SHU9119. The observation that the maximized responses in food and water intake during i3vt SHU9119 treatment were similar in pregnant and non-pregnant rats suggests a lack of additional orexigenic neuronal systems during pregnancy acting independent from—or parallel to—the orexigenic drives mediated by inhibited brain MC activity. With respect to body weight gain, a different picture emerged. Despite the similarities in ingestive behaviour in i3vt SHU9119-treated pregnant and non-pregnant females, body weight gain in pregnant females was much stronger than in non-pregnant females treated with SHU9119. This implies that inhibition of MC signalling in pregnant rats leads to a higher level of food efficiency relative to that observed in non-pregnant rats, which does suggest an unknown mechanism acting independently of MC receptor signalling. A similar comparison between SHU9119-treated lactating and non-lactating animals is more difficult, as we cannot exclude the possibility that the higher body weight of SHU9119-treated non-lactating rats relative to SHU9119-treated lactating rats may have caused a relatively lower orexigenic drive in the first group, for example through increased negative feedback inhibition by leptin. This could be relevant as plasma leptin levels are normally suppressed during lactation in rats.[39–42] Together with an increased expression of some of the short forms of the leptin receptor (Ob-Re and Ob-Rf) in the hypothalamus[43] and an increased leptin-binding activity in plasma,[41] this could have accounted for the suppression of MC signalling during lactation as well. An additional explanation may be that AgRP (as opposed to the 'passive' receptor blocker SHU9119) acts as an inverse agonist on MC4 receptors,[44] which could gate other orexigenic systems post-synaptically to maximize ingestive behaviour. These could include, for example, NPY in projections arising not only

from ARC neurones but also from the dorsomedial hypothalamus to other food intake-regulating areas.[45] A role for reduced MC signalling has been proposed for the latter, as novel expression of NPY in DMH has been observed not only in MC4R null mice[46] but also in other rodent models of hyperphagia such as the diet-induced obese (DIO) mouse.[47]

At birth, the litter weight of SHU9119-treated mothers was reduced compared with saline-treated mothers, an effect owing to a lower litter size, but not to reduced individual pup weight. We do not know the mechanism underlying this effect. Although the success rates of pregnancies of SHU9119-treated (67%) and saline-treated (63%) rats were about equal, it may be possible that some embryos were absorbed during the pregnancies of SHU9119-treated mothers, or that they were cannibalized right after birth and before counting. Although a direct effect of SHU9119 on fetal development by placental passage cannot be excluded entirely, this possibility is highly unlikely because the molecular properties of cyclic α-MSH analogues prevents the easy passage of these compounds across the blood–brain barrier to the peripheral circulation in the infused mothers.[48] Over the course of lactation, SHU9119-treated mothers were able to rear their pups equally well as saline-treated mothers, indicating that reduced MC signalling does not negatively affect mother–pup interactions. In fact, body weight increased transiently in pups of SHU9119-treated mothers at day 16 relative to controls. These effects contrast highly with those found with central infusion of NPY during pregnancy and lactation, which causes a very strong suppression of pup growth.[49] Although NPY may function to adapt maternal metabolism to the energy demands of nursing offspring,[37] an overpowering increase of NPY may result in an abrupt ending of lactation.[49] The physiological function of this may be to protect a mother from starvation if there is not enough food for both herself and her offspring during periods of famine. Apparently, low MC activity during pregnancy and lactation acts in a different way, not diverting fuels away from fetal development. Following weaning, body weight gain became more pronounced in male offspring of SHU9119-treated mothers relative to male offspring from saline-treated mothers with a final increase of 7.1% at 9 months of age. In females, only a treatment effect was found, and at 9 months of age the differences were lost. Viewing the relative weights of organs and tissues of male offspring in different treatment groups indicated that all (except for the weight of the full gastrointestinal tract) contributed to this weight gain but that the relative contributions of retroperitoneal (+36%) and epididymal (+20%) fat pads were found to be the strongest. Increases in visceral fat stores are often associated with other hallmarks of the metabolic syndrome, such as insulin resistance and glucose intolerance,[50] but we detected no differences in glucose and insulin responses between offspring of SHU9119-treated and saline-treated mothers during OGTT and IVGTT.

Although body weight was clearly different in male offspring of mothers in the two treatment groups, the general linear model in which litters were nested in treatments did not attain significant differences between these or any other tissue or organ weights of the different treatment groups. It might be that a part of this is explained by the relatively small number of pregnancies observed in vehicle and SHU9119-treated groups. On the other hand, the effect of 'litter' in the general linear model was significant in most cases, indicating that differences between litters within treatment groups were relatively large compared to differences between litters in different treatment groups. To investigate the role of 'litter,' we averaged various tissue and organ weights per litter as dependent factors, and performed a backward regression analysis in which we included gestational body weight gain, treatment group as well as pre-gestational body weight as independent factors. The latter effect can be imagined if body weight and its proportions are regulated traits that can be passed on through genes from one generation to the next.[51,52] Consistent with a relatively large contribution of differences in retroperitoneal fat pads to the observed differences in body weight between offspring from different treatment groups was the finding that the gestational weight gain of mothers was the strongest contributor to the weights of visceral fat pads in male offspring. To our surprise, however, we observed that the pre-gestational body weight of mothers was the strongest contributor to weights of subcutaneous fat, carcass and various structural organs, including the liver and the total body weight of male offspring at 9 months of age. In female offspring, only gestational weight gain—and not the pre-gestational body weight—contributed significantly to weights of tissues and organs at 9 months of age. Although these effects are very significant in females as well, they are less conspicuous than in males as body weights of female offspring from SHU9119-treated and saline-treated mothers were not significantly different.

There is a large literature on the mechanisms that regulate energy balance. By definition, these include factors involved in the control of ingestive behaviour and energy expenditure. An example is the brain MC system, which, upon reduction of MC4 stimulation, will lead to an increase in food intake and to a reduction in energy expenditure as seen in this study. Besides increased food intake, a reduction in energy expenditure might have played a role in augmenting body weight gain in i3vt SHU9119-treated mothers as well. We observed that male offspring of SHU9119-treated rats had an increased resting metabolic rate (RMR) and daily energy expenditure (DEE) compared with control males. As differences were entirely lost when RMR and DEE were calculated per gram body weight, this indicates that these factors are within the normal physiological range. Apparently, the trait of high food efficiency induced by low MC signalling as seen in the mothers is not passed on to the offspring. In fact, using backward regression analysis, we found that the pre-gestational body weight, but not the gestational body weight gain, was a stronger contributor to DEE, which could correspond to the contribution of pre-gestational body

weight to increased weights of metabolically active tissues in male offspring. Gestational weight gain, on the other hand, contributed greatly to carbohydrate oxidation, and this effect was even more pronounced in female offspring. In the latter event, a significant inverse contribution of gestational weight gain was found on fat oxidation in female offspring as well, indicating a shift from fat oxidation to carbohydrate oxidation. Although the underlying mechanisms have yet to be discovered, these influences of gestational body weight gain on fuel oxidation could have dramatic consequences for the offspring when they are challenged with a high fat diet. At the time of indirect calorimetry, we also assessed individual food intake and water intake in the isolated offspring. Although we anticipated an increased intake in the offspring from SHU9119-treated mothers to match the increase in RMR and DEE, we did not detect any difference. As animals were normally housed 2–3 per cage, it might be possible that the temporary individual housing conditions masked the effects of maternal SHU9119 treatment to increase ingestive behaviour.

In summary, the blockade of brain melanocortin receptors during pregnancy and lactation is able to cause gestational weight gain, which programmes increased body weight gain in male offspring rats and, to a lesser extent, in female offspring rats. The degree of gestational weight gain may selectively programme increased visceral adipose tissue storage in male offspring, and these effects could be linked to increased CHO oxidation relative to oxidation of fats. On the other hand, maternal factors already present before pregnancy may contribute to a structural increase of body weight in male offspring. Apparently, obesogenic factors such as feeding a high-energy diet, chronic psychological stress and/or genetic background are much stronger risk factors for fetal programming of metabolic derangements than gestational weight gain *per se*.

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